SYNOPSIS

Drug Release Analysis and Anticancer Potential of *Tabebuia pallida* Silver Nanoparticles Loaded Liposomes against MOLT-3 Cells - *in vitro*

PRIYANKA J

(Ph.D Biochemistry)

18PHBCF005

Under the supervision of

Dr. R. Nirmaladevi

Assistant Professor of Biochemistry

Department of Biochemistry, Biotechnology and Bioinformatics

Submitted to

Avinashilingam Institute for Home Science and Higher Education for Women

Coimbatore 641043

October 2021

Introduction

Cancer is an abnormal cell growth that is considered as the prime cause of mortality worldwide and it decreases the life span of people in all the countries. According to the survey of World Health Organization in the year 2019 it is estimated that the cancer is the first or second leading cause of death in 112 of 183 countries and it is in the third or fourth position in the cause of mortality in 23 countries (WHO, 2020). Leukaemia is a type of cancer which leads to the increase in the number of leucocytes either in the bone marrow or in blood. There are four different types of leukaemia based on its origin, pathogenesis and prognosis. They are Chronic Lymphocytic Leukemia (CLL), Chronic Myeloid Leukemia (CML), Acute Lymphoblastic Leukemia (ALL) and Acute Myeloid Leukemia (AML) (Kipps *etal.,* 2017). The most common type of cancer among children is Acute Lymphoblastic Leukaemia and it accounts for about 25% of all cancers in children who are below the age of 15. In most of the cases, ALL is treatable only in the case of developed countries. Developing countries are still facing difficulty in treating ALL due to the lack of facilities to carry out modern treatment strategies (Abdelmabood *etal.,* 2020).

Over the last few years, various anticancer drugs or agents are deployed for cancer management which includes hormone therapeutics, alkylating agents, biological response modifiers, antibiotics, platinum compounds, antimetabolites and mitotic inhibitors. The major disadvantage of these anticancer agents is that their adverse effects, which include fatigue, immunosuppression, liver damage, severe nausea and vomiting, lack of appetite, abdominal pain, back pain and weakness. Therefore an anticancer drug with minimal or no side effects is the need of the hour (Sagbo and Otang, 2021)

Ancient ayurveda and Indian traditional medicine uses plants for the treatment of several ailments. The plants possess medicinal properties due to the presence of abundant phytochemicals and their derivatives. One of the important properties of these phytochemicals is that they can act as anticancer agents. These phytochemicals are biologically active compounds that are present in nature with considerable anticancer potential. The major advantage of these phytochemicals is that they produce very less or minimal side effects. So the purification of these phytochemicals from the plants and using them as anticancer drugs will be the best adverse effect free treatment for cancer (Choudhari *etal.,* 2020).

Recently silver nanoparticles have gained much importance among the scientific community because of their diverse therapeutic properties. Enormous and less expensive methods are available for the synthesis of silver nanoparticles including chemical, physical, electrochemical and sonochemical methods (Khandel *etal.,* 2018; Fahmy *etal.,* 2019). But the major disadvantage of these methods is that they are hazardous to the environment especially when the synthesis is carried out with the use of chemicals and also the silver nanoparticles synthesized using these methods will tend to agglomerate easily because of their monodispersity (Gulati *etal.,* 2018). Among the various methods available for the synthesis of silver nanoparticles, the plant mediated green synthesis is found to be very effective, eco- friendly, easy to carryout and cost effective. The phytochemicals present in the plant extracts acts as a capping and reducing agent that prevents them from agglomeration and this makes them very stable for a longer period (Bukhari *etal.,* 2019). These silver nanoparticles are used as anticancer cancer agents due to their antiproliferative and apoptosis inducing properties (Almashgab *etal.,* 2020)

Liposomes are spherical vesicles made up of lipid bilayer enclosed in hydrophilic core (Jain *etal.,* 2018). They can act as an effective drug delivery carrier. The major advantage of these liposomes is that they can incorporate both hydrophilic and hydrophobic therapeutic agents (Jain *etal.,* 2019). The other advantages of these liposomes is that they are safe, effective, possess cell like membrane structure, enhanced life span, biocompatible, efficiently protects and delivers the active groups of the drug (Li *etal.,* 2019)

Tabebuia pallida often called as "Pink trumpet tree" that belongs to the family "Bignoniaceae" is one of the largest genera that is distributed in South America, Central America and Western region of India (Bussmann, 2018). It is usually grown as an ornamental tree that reaches upto 25-30 meter height. *Tabebuia pallida* is the candidate plant of the current study to synthesize the silver nanoparticles. *Tabebuia* species are mainly used in Ancient folk medicine for treating various diseases including allergies, syphilis, constipation, malaria, prostatitis, cutaneous infections, diabetes, stomach disorders, depression, cancer, irritability, inflammation, poor memory, pain, anxiety, fungal and bacterial infections (Ferraz *etal,* 2017 and Regalado *etal.,* 2017)

Thus, this present study aims to synthesize and characterize the silver nanoparticles and silver nanoparticles loaded liposomes from *Tabebuia pallida* leaf extract and to analyze it's *in vitro* drug release profile, antioxidant and anticancer potential.

Objectives

- To synthesize and characterize AgNPs and AgNPs loaded Liposomes from *Tabebuia pallida* leaf extract
- To evaluate the *in-vitro* drug release of AgNPs loaded Liposomes and to validate through mathematical models
- To evaluate antioxidant and anticancer potential of AgNPs of *T. pallida* leaves and their liposomes

The present study aims to synthesize silver nanoparticles loaded Liposomes and to analyze their drug release profile, antioxidant and anticancer potential using acute lymphoblastic leukaemia cells (MOLT-3) and peripheral blood lymphocytes (Normal cells). The study was designed as four different phases.

Phase I

Synthesis and Characterisation of Silver nanoparticles and Silver nanoparticles loaded Liposomes from *Tabebuia pallida* leaf extract

Phase II

Evaluation of Drug Release of Silver nanoparticles Loaded Liposomes and their validation using various mathematical models

Phase III

Evaluation of Antioxidant and free radical scavenging Potential of Silver nanoparticles and Silver nanoparticles Loaded Liposomes of *Tabebuia pallida*

Phase IV

Evaluation of Anticancer potential of Silver nanoparticles and Silver nanoparticles Loaded Liposomes of *Tabebuia pallida*

Results

Phase I

The phase I of the study aims to synthesize and characterize silver nanoparticles and silver nanoparticles loaded liposomes from *Tabebuia pallida* leaf extract. As a preliminary study we have carried out the Soxhlet Extraction of *Tabebuia pallida* leaves using 7 different solvents (Methanol, Ethyl Acetate, Benzene, Ethanol, Chloroform, Petroleum Ether, Acetone, and Water) and conducted Free radical Scavenging assays like DPPH radical scavenging assay, ABTS radical scavenging assay, Hydroxyl radical scavenging assay, Hydrogen Peroxide Radical Scavenging Assay, Reducing power assay and Nitric oxide radical scavenging assay. From the results of free radical scavenging assays it was found that the ethanol and Water extract was found to be best radical scavengers. So we have carried out the Soxhlet extraction using various proportion of these solvents (Ethanol 20: Water 80, Ethanol 80: Water 20, Ethanol 60: Water 40, Ethanol 40: Water 60 and Ethanol 50: Water 50) and carried out Sunlight mediated Green Synthesis of Silver nanoparticles.

The synthesized silver nanoparticles were analysed using UV-Visible spectrophotometer to identify the best extract for the synthesis of silver nanoparticles. Usually the silver nanoparticles are known to exhibit an UV–Visible absorption maximum in the range of 400–500 nm because of their surface plasmon resonance. A good and little narrowed peak at 450 nm was obtained for Ethanol 60: Water 40 extract, noise and broadened peaks were observed for other extracts which indicates that the Ethanol 60: Water 40 extract was the best for AgNP synthesis. Therefore Ethanol 60: Water 40 extract was used for the further analysis. The elemental mapping of the synthesized AgNPs was done using EDAX APEX. Various elements like Ag, Cl, C, and O are present in the synthesized nanoparticles and their percentage weight is 65.94, 3.52, 15.46, and 15.08 respectively. The XRD analysis was carried out to identify the structure and nature of the synthesized AgNPs. XRD pattern of the green synthesized *Tabebuia pallida* nanoparticles shows various diffraction peaks at 2θ = 32.20, 38.08, 46.18, 54.80 corresponds to the (111), (200), (120) and (202) planes respectively. These diffraction patterns indicate the presence of face centered cubic crystalline structure of the synthesized nanoparticles.

The silver nanoparticles loaded liposomes were synthesized using thin film hydration method coupled with sonication with the use of a triglyceride lipid lecithin and cholesterol in the molar ratio 2:1. The encapsulation efficiency of the AgNPs loaded liposomes was found to be 82.25%. FTIR analysis was carried out to identify the functional groups that are responsible for the synthesis of Silver nanoparticles and Silver nanoparticles loaded liposomes. Various functional groups were identified in the FTIR spectrum which indicates that the phytochemicals present in the plant extract acted as a capping agent in the formation of silver nanoparticles. The size of the synthesized silver nanoparticles and silver nanoparticles loaded liposomes were analysed using Field Emission Scanning Electron Microscopy. Usually the size of the nanoparticles ranges between 1 to 100nm. The synthesized nanoparticles were found to be uniformly spherical in shape with the size ranging between 31.76 nm – 50.36 nm and the size of the AgNP loaded liposomes are found to be ranging between 80-97 nm and are also spherical in shape. From the results of Dynamic Light Scattering analysis, it is observed that the average size of the silver nanoparticles and silver nanoparticles loaded liposomes was 55.4 nm and 172.1 nm with a PolyDispersity Index of 0.33 and 0.381 respectively. This Polydispersity index value indicates that the synthesized silver nanoparticles and silver nanoparticles loaded liposomes were stable and will not undergo agglomeration for a longer period. The Zeta potential of the green synthesized silver nanoparticles was found to be -15.6 mV. The zeta potential of the AgNP loaded liposomes was found to be -21.5 mV. From this negative zeta potential it could be confirmed that the synthesized silver nanoparticles and silver nanoparticles loaded liposomes will be stable for a longer duration since there will be a strong repulsion among the particles that prevents them from agglomeration. The thermal stability of AgNPs and AgNP loaded liposomes were analysed using Thermo Gravimetric Analysis and it was found that the stability decreases with increase in temperature.

Phase II

The phase II of the study was designed to evaluate the *in vitro* drug release profile of silver nanoparticles loaded liposomes and validate it through various mathematical models. The *in vitro* drug release study was carried out in three different pH like pH 5.5 which is similar to the pH of mature endosomes of cancer cells, pH 6.8 which is similar to the pH of cancer cells and pH 7.4 which is similar to the pH blood. A high AgNP release was found at a pH of 5.5

when compared to the others which clearly indicates the targeted delivery of the AgNPs by the lipsomes.

This drug release profile was validated using different mathematical models including Zero order model, First order model, Higuchi model, Korsmeyer and Peppas model and Hixson Crowel model. A graph is plotted between cumulative drug release % and Time to analyze whether the drug release follows Zero order model. It is observed from the graph that the drug release of AgNPs from the liposome does not follow perfectly the principle of zero order release kinetics because of their less r^2 value. Similarly a graph was plotted between log cumulative % of drug remaining against time to understand whether the drug release follows first order model. The graph plotted reveals that the drug release of AgNP from the liposome does not follow the principle of first order release kinetics as there is lower value of coefficient of correlation.

Thirdly the graphical representation of cumulative % of drug release against square root of time represents that drug release of AgNP from the liposome is perfectly following Higuchi drug release model as the drug release profile is very closest to trend line or regression line and there is highest value of coefficient of correlation. This indicates that the AgNPs release from liposomes follows diffusion. Thus it can be concluded that silver nanoparticles release from liposomes follows diffusion as per the observations of Higuchi model. Further to know the type of diffussion occurred during the drug release process Korsmeyer and Peppas model was performed. For this a graph was plotted between the log cumulative % of drug release against log time. From the release exponent value obtained in the graph it was confirmed that the drug release follows non-fickian transport and is time dependent.

Phase III

The phase III of the study was designed to evaluate the antioxidant potential of hydro ethanolic extract, AgNPs and AgNP loaded Liposomes. Free radical scavenging and antioxidant potential of the hydro ethanolic extract, AgNPs and AgNP loaded Liposomes were evaluated using DPPH radical scavenging assay, ABTS radical scavenging assay, Hydroxyl radical scavenging assay, Reducing Power assay, Hydrogen peroxide radical scavenging assay and Nitric oxide radical scavenging assay compared with the standard antioxidant quercetin. From these assays, it was found that the hydro ethanolic extract, AgNPs, AgNP loaded Liposomes and

standard quercetin exhibited a dose-dependent scavenging potential. When the concentration of both standard and the samples increased the radical scavenging ability was found to be increased to a greater extent. Among the various samples tested, the Silver nanoparticles loaded liposomes possess high radical scavenging potential.

Phase IV

The cell viability and anticancer activity against MOLT-3 cells of the hydro ethanolic extract, AgNPs and AgNP loaded Liposomes were analyzed in this phase. Peripheral Blood Lymphocytes (PBL) was used as the normal counterpart for the MOLT-3 cells. From MTT dye reduction assay and Sulphorhodamine B assay, it was found that the liposomes are very effective against MOLT-3 cells and doesnot affect the normal PBL cells much which indicates that the various treatment groups are not cytotoxic to the normal cells. Measurement of Apoptosis was carried out using Annexin V/FITC staining. In the control group, all the cells are in the live phase and in the treated cells, the cells are in both early and late apoptotic phase which clearly indicates the cytotoxicity of the treatment groups. In the case of silver nanoparticles loaded liposomes, the cells are in late apoptosis which confirms the high cytotoxic effect of the liposomes against MOLT-3 cells. In the case of normal PBL most of the cells are in live phase in control and in the treated cells some of the cells are in early and late apoptosis which is considerably less when compared to MOLT-3 cells of leukemic origin. In the case of liposomes 94% of the cells are in live phase which clearly depicts the targetted drug delivery by the liposomes to the leukemic cells.

Mitochondrial Membrane Potential of the control and treated cells were analysed using JC1 Staining. If the mitochondria are intact with normal membarane potential, the JC1 dye enters into the mitochondria and exhibit red coloured fluorescence. If there is a loss of mitochondrial membrane potential disappearance of red fluorescence will be observed. After treating with MOLT-3 cells with hydroethanolic extract, AgNPs and AgNP loaded liposomes, there was a loss of mitochondrial membrane potential to a great extent was observed which was confirmed by the formation of more monomers. In the case of normal peripheral blood lymphocytes, minimal loss of mitochondrial membrane potential was observed, which was confirmed by the presence of more J-aggregates.

Changes in the phases of cell cycle were analysed using flow cytometer. For MOLT-3, In the case of untreated control the cells are disbursed in all the phases of cell cycle. But in the treated cells with hydroethanolic extract, AgNPs and AgNP loaded liposomes; the cells were arrested in G0-G1 phase. In the untreated PBL control, cells were distributed in all the phases of cell cycle. Similar results were observed in the treated PBL cells which clearly indicate that the treatment groups do not produce considerable cytotoxic effect on the normal counterpart. Measurement of ROS Production was analysed using flow cytometer. From ROS assay, it is evident that the various treatment groups in MOLT-3 cells transferred the DCF fluorescence graph from M2 to M1. This transfer indicates that there is a reduction in ROS production by the various treatment groups. In the case of PBL more cells are in M1 which represents that there is a less ROS production.

Conclusion

From the present study it was found that the green synthesized *Tabebuia pallida* silver nanoparticles loaded liposomes were found to possess both antioxidant and anticancer potential. The targeted drug delivery of silver nanoparticles by liposomes to the MOLT-3 cells was clearly identified from the drug release assay. Therefore *Tabebuia pallida* silver nanoparticles loaded liposomes can be used as a candidate for the development of new anticancer drug.

References

1. Abdelmabood, S., Abdelmabood, S., Fouda, A.E., Boujettif, F. and Mansour, A. (2020) Treatment outcomes of children with acute lymphoblastic leukemia in a middle-income developing country: high mortalities, early relapses, and poor survival, Jornal de Pediatria, 96(1), 108–116.
2. Almashgab, A.M., Yahya, E.B. and Banu, A. (2020) The Cytotoxicity Effects of Outer Membrane Vesicles Isolated From Hospital and Laboratory Strains of Pseudomonas Aeruginosa on Human Keratinocyte Cell Line. Malaysian Journal of Science, 39 (3): 45 - 53 .
3. Bukhari, N., Joseph, J.P., Hussain, S.S., Khan, M.A., Wakim, M.J. amd Yahya, E.B. (2019) Prevalence of Human Papilloma Virus Sub Genotypes following Head and Neck Squamous Cell Carcinomas in Asian Continent, A Systematic Review Article. Asian Pacific Journal of Cancer Prevention, 20 (11), 3269-77
4. Bussmann, R.W. (2018) *Tabebuia avellanedae Lorentz* ex Griseb, Medicinal and Aromatic Plants of South America. Springer, 439-451
5. Choudhari Amit, S., Mandave Pallavi, C., Deshpande Manasi, Ranjekar Prabhakar and Prakash Om (2020) Phytochemicals in Cancer Treatment: From Preclinical Studies to Clinical Practice, Frontiers in Pharmacology, 10, 1614.
6. Fahmy, H. M., Mosleh, A. M., Elghany, A. A., Shams-Eldin, E., Serea, E. S. A., Alia, S. A. and Shalan, A.E. (2019). Coated silver nanoparticles: synthesis, cytotoxicity, and optical properties. RSC Adv., 9, 20118.
7. Ferraz-Filha, Z.S., Ferrari, F.C., Araújo, M.C.D.P.M. and Bernardes, A.C.F.P. (2017) Effects of the aqueous extract from *Tabebuia roseoalba* and phenolic acids on hyperuricemia and inflammation. Evid. Based Complement. Altern. Med., 2017, 1-10.
8. Gulati, S., Sachdeva, M. and Bhasin, K. K. (2018) Capping Agents in Nanoparticle Synthesis: Surfactant and Solvent System. AIP Conference Proceedings, 1953.
9. Jain, A., Hurkat, P. and Jain, S.K. (2019) Development of liposomes using formulation by design: basics to recent advances. Chem. Phys. Lipids, 224, 104764.

10. Jain, A., Kumari, R., Tiwari, A., Verma, A., Tripathi, A., Shrivastava, A. and Jain, S.K. (2018) Nanocarrier based advances in drug delivery to tumor: an overview. Curr. Drug Targets, 19 (13), 1498–1518.
11. Khandel, P., Yadaw, R. K., Soni, D. K., Kanwar, L. and Shahi, S. K. (2018). Biogenesis of metal nanoparticles and their pharmacological applications: present status and application prospects. *J. Nanostruct. Chem,*. 8, 217–254.
12. Kipps, T.J., Stevenson F.K., Wu, C.J., Croce, C.M., Packham, G., Wierda, W.G., O'Brien, S., Gribben, J. and Rai, K. (2017) Chronic lymphocytic leukaemia, Nat Rev Dis Primers, 3, 16096.
13. Li, M., Du, C., Guo, N., Teng, Y., Meng, X., Sun, H., Li, S., Yu, P. and Galons, H. (2019) Composition design and medical application of liposomes. Eur. J. Med. Chem., 164, 640–653.
14. Regalado,A.I., Mancebo, A., Paixão, Y., López, N., Merino, Sánchez. L.M. (2017) Antinociceptive activity of methanol extract of *Tabebuia hypoleuca* (C. Wright ex Sauvalle) Urb. Stems. Med. Princ. Pract., 26, 368-374
15. Sagbo, I.J. and Otang-Mbeng, W. (2021) Plants Used for the Traditional Management of Cancer in the Eastern Cape Province of South Africa: A Review of Ethnobotanical Surveys, Ethnopharmacological Studies and Active Phytochemicals, Molecules, 26, 4639.
16. World Health Organisation (WHO) (2020) Global Health Estimates 2020: Deaths by Cause, Age, Sex, by Country and by Region, 2000-2019.